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Methods for the Detection and Estimation of Numbers of *Salmonella* in Dried Eggs and Other Food Products[1]

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Dehydrated eggs are an important item of procurement for Armed Forces feeding. Because the presence of *Salmonella* in egg products constitutes a potential health hazard, it is obviously important to the Armed Forces that food infections and carrier states caused by *Salmonella* in eggs be prevented among troops. The purpose of this report is to describe improved test methods for estimating the extent of *Salmonella* contamination in foods in order to assist in the control and elimination of this food infection hazard. Many investigators (Gibbons and Moore, 1944; Schneider, 1946; Gibbons, 1947; Solowey *et al.*, 1947; Solowey and Rosenstadt, 1948) have reported the occurrence of these microorganisms in eggs, and McCullough and Eisele (1951*a*, 1951*b*, 1951*c*, 1951*d*) established the pathogenicity for humans of strains of *Salmonella* derived from spray-dried whole egg. Outbreaks of *Salmonella* infections which were traced to eggs have been described (Watt, 1945; Mitchell *et al.*, 1946; Medical Research Council, 1947). Edwards *et al.* (1948) state that "eggs and food products containing eggs may more often be the medium of transmission of *Salmonella* from animals to man than any other animal food product." Hinshaw and McNeil (1951) in a review of *Salmonella* infection as a food industry problem stress the importance of animal and human reservoirs of the infection, and state that "the genus *Salmonella* is one of the important causes of the infection type of food poisoning."

The isolation and identification of members of the *Salmonella* group from food products present many difficulties. Various methods and modifications, many of which were originally developed for isolating pathogens from feces and sewage, have been proposed throughout the years (Leifson, 1936; Hynes, 1942; Galton and Quam, 1944; Felsenfeld, 1945; McCullough and Byrne, 1952; Ayres, 1953). As a result of these investigations, the use of selective enrichment media is a common procedure and is preferred to direct culture for the isolation of *Salmonella* from suspected materials which contain a large and varied population of microorganisms.

MATERIALS AND METHODS

We have attempted to overcome some of the deficiencies in the recommended procedures by using the dilution-enrichment-subculture method to be described. The primary purpose of the method is to determine the degree of *Salmonella* contamination and the changes in numbers of these organisms during processing and storage of eggs and egg products.

Twenty grams of egg powder are weighed aseptically in a flask containing 180 ml of sterile distilled water and a tablespoonful of glass beads. The egg suspension

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is shaken by hand intermittently for 1 hour at room temperature. This preliminary soaking period is followed by a second hour of vigorous mechanical shaking at 150 strokes per minute to insure homogeneity. In our experience, there has been no increase in *Salmonella* during this 2-hour period.

A 1:100 suspension is prepared from the thoroughly emulsified and reconstituted egg by diluting 2.5 ml of the 1:10 suspension with 22.5 ml of sterile distilled water and decimal serial dilutions are prepared in a similar manner. Twenty ml aliquots of the 1:10 (equivalent to 2 g of egg solids) are pipetted into 5 replicate tubes containing 20 ml of double strength Selenite-F broth (BBL) to which has been added 10 mg of cystine per liter. Two ml aliquots of the 1:10, 1:100, 1:1000, and 1:10,000 dilutions (equivalent to 0.2, 0.02, 0.002, and 0.0002 g of egg solids, respectively) are pipetted into 5 replicate tubes containing 40 ml of single strength Selenite-F broth with added cystine (10 mg per liter).

The enrichment cultures are incubated 18 to 20 hours at 37 C, following which a loopful from each tube is streaked onto brilliant green agar (Difco) and bismuth sulfite agar (BBL) plates which are incubated 24 and 48 hours, respectively.

Single, well isolated colonies suspected of being *Salmonella* are picked from each plate and transferred to triple sugar iron agar (Difco) (TSI) slants and these are incubated 24 hours at 37 C. Those cultures showing characteristic *Salmonella* reactions on TSI are gram-stained and tested for motility. They are also examined for their ability to ferment sugars, produce urease and indol, and agglutinate in *Salmonella* polyvalent and group antisera (Lederle).[3]

It is often necessary to know the degree of *Salmonella* contamination during stages of processing of eggs in order to check the efficiency of the pasteurization treatment. For this purpose, most probable numbers (MPN) are determined from the significant number of *Salmonella*-positive broth tubes in the appropriate three highest dilutions. Numerical values are taken from MPN tables in *Standard Methods for the Examination of Water and Sewage* (APHA, 1946). The general rules applying to the use of most probable number tables are followed as illustrated in the sample calculation shown in table 1.

The Significant Number in this sample is 531. The corresponding MPN present in 0.2 gram portions (obtained from the probable numbers table) equals 110 multiplied by a factor of 0.1. Thus, $\frac{(110)\ (0.1)}{0.2} = 55$ per gram of egg powder as the MPN value. The factor of 0.1 is necessary to convert the MPN values in the table from an MPN per 100 ml basis for which it was devised to an MPN per gram basis adaptable to the present determination.

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TABLE 1

Sample Inoculated into Selenite Broth Tubes (5 Replicates)		Equivalent Weight of Egg Solids per Tube	Number of Tubes *Salmonella*-Positive out of 5 Tubes
Volume	Dilution	Grams	
ml			
20	1:10	2.0	5
2	1:10	0.2	5
2	1:100	0.02	3 *
2	1:1000	0.002	1
2	1:10,000	0.0002	0

* Significant number = 531.

TABLE 2. *Frequency and extent of Salmonella content in processing dried eggs during a 1954 procurement*

	Raw Liquids	Pasteurized Glucose-Free Liquids	Glucose-Free Powders
No. of samples examined	51	39	113
No. positive for *Salmonella*	51 (100%)	14 (35.8%)	6 (5.3%)
No. positive on BG* agar	34 (66.6%)	4 (10.2%)	6 (5.3%)
No. positive on BiS† agar	51 (100%)	14 (35.8%)	6 (5.3%)
Median *Salmonella* MPN BG agar	12.2/g‡	0.38/g‡	0.1/g§
Median *Salmonella* MPN BiS agar	2,600/g	0.85/g	0.1/g
Antigenic groups isolated	D-E-C_1	E-C_1	E-C_1

* Brilliant green agar.
† Bismuth sulfite agar.
‡ Egg solids.
§ Powder.

This method for determining the MPN of *Salmonella* has been used effectively in the examination of raw and pasteurized liquid whole egg, dehydrated whole egg, egg albumen, both liquid and dried, and liquid egg yolk. For liquid products the samples are prepared by suspending 20 grams in 180 ml of sterile water, and the resultant 1:10 dilutions are examined in the above described manner. In such cases it is necessary to know the solids content of the product if *Salmonella* MPN values are reported on a "per gram of solids" basis.

RESULTS AND DISCUSSION

The quantitative procedure has been employed extensively in our laboratory for several years in the examination of dried eggs purchased for the Quartermaster Corps, and various analytical modifications have been tested and compared. The method as evolved has been applied to studies of the *Salmonella* incidence in commercially broken-out liquid whole egg for use in spray drying and to studies of the effectiveness of various pasteurization treatments for the elimination of *Salmonella* from dehydrated whole egg. A typical study, illustrated in table 2, presents data obtained from 203 process samples during a 1954 procurement

TABLE 3. *Salmonella recoveries from three enrichment broths using egg albumen samples with varying numbers of Salmonella*

	MPN 5/gm	MPN 85/g	MPN 8000/g
Selenite-F with cystine	20/84* = 24%	30/84 = 36%	41/48 = 85%
Selenite-F	0/84	20/84 = 25%	34/48 = 71%
Tetrathionate	3/48 = 6%	0/48	12/48 = 25%

* Fraction indicates the following:
Numerator = Number of *Salmonella* positive plates.
Denominator = Number of plates streaked.

for the Armed Forces of dehydrated whole egg. Frozen samples of the raw liquid egg and glucose-free pasteurized liquid egg, and samples of the finished egg powder were obtained from a commercial processing plant for determinations of the *Salmonella* MPN values.

The results obtained using brilliant green agar plates have been compared with those obtained using bismuth sulfite agar plates. It is obvious from the data shown that the bismuth sulfite agar was more effective than brilliant green agar for the isolation of *Salmonella* from liquid eggs, especially from the liquid before pasteurization. *S. pullorum* was found only in the raw liquid samples, and it is generally agreed that bismuth sulfite agar supports the growth of *S. pullorum* better than brilliant green agar. There is an abundant bacterial population in raw liquid egg which is not held back as effectively on brilliant green agar as on the more inhibitory bismuth sulfite agar and consequently the *Salmonella* are overgown. It is generally accepted that employment of more than one selective or differential plating medium increases the chances of isolating *Salmonella*. For example, in the examination of 90 samples of liquid whole egg, 5 samples showed MPN values which were higher when the two agars were used than when either agar was used alone.

The validity of the dilution-enrichment-subculture method described for estimating the most probable numbers of *Salmonella* in eggs was established by the serological identification of presumptive-positive cultures at a *Salmonella* Typing Center. Of 1,250 isolates, 1,246 or 99.67 per cent were found to be *Salmonella* and 4 were *Paracolobactrum intermedium*, Bethesda group.

In certain instances modifications of the dilution-enrichment-subculture method have been made. For example, in screening a large number of samples a qualitative test for the presence of *Salmonella* is used and, if positive, is followed by the quantitative procedure. In the case of egg powder samples, 10 g of the egg are weighed aseptically in a flask containing 50 ml of Selenite-F enrichment broth. After 18 to 20 hours' incubation, the cultures are streaked onto brilliant green and bismuth sulfite agar plates. When precooked frozen meals are screened, 50 ml of a 1:5 suspension of the food (equivalent to 10 g of food) are pipetted into 200 ml of selenite broth. After 18 to 20 hours' incubation the cultures are streaked onto brilliant green and bismuth sulfite agar plates which are examined after 24 and 48 hours' incubation, respectively. Approximately 150 components of precooked frozen meals, including meats, vegetables, and potatoes, were examined for *Salmonella* by this method and all were found to be free of *Salmonella*.

Another product for which a qualitative method has been developed is dried egg albumen. Since albumen-containing meringue powder is used primarily in confections and is often uncooked or insufficiently cooked to kill pathogenic bacteria, it is important that the product be free of *Salmonella*.

North and Bartram (1953) reported that the addition of cystine to Selenite-F broth enhanced the growth of *Salmonella*. Consequently, a comparison was made of Selenite-F, Selenite-F with added cystine, and tetrathionate broths using as inocula three dried egg albumen samples of known *Salmonella* contamination. Most probable numbers of *Salmonella* had been determined earlier with the dilution-enrichment method previously described. One sample had an MPN of 5 *Salmonella*/g, another 85/g, and the third 8,000/g. The specific types isolated were *S. oranienburg*, *S. kentucky*, and *S. pullorum* from sample 1; *S. montevideo* and *S. oranienburg* from sample 2; and *S. tennessee* and *S. pullorum* from sample 3. Five g of egg albumen were weighed aseptically and put into 50 ml of Selenite-F, tetrathionate, and Selenite-F broth containing a cystine concentration of 10 mg per liter. After incubation and subsequent streaking on bismuth sulfite, brilliant green, *Salmonella-Shigella* (S-S) (Difco) and MacConkey's (Difco) agar plates, it was found that the greatest number of positive *Salmonella* cultures was obtained from Selenite-F with added cystine. Data confirming North and Bartram's observations on enhanced growth with added cystine in selenite broth are presented in table 3. Therefore, cystine has been incorporated routinely in our Selenite-F medium and was present in the selenite broth in the test methods which have been described.

Still another food product which has been examined for *Salmonella* is inactive dried yeast. A method suggested for this product by the Food and Drug Administration, which involves the incubation of a 1:5 yeast suspension for 24 hours at 30 C before it is put into selenite and tetrathionate broths, gives good qualitative results. We attempted with one lot of yeast to obtain an MPN of *Salmonella* following the method described for eggs, but the results were negative. When the 1:10 yeast slurry was incubated for 24 hours at 30 C before inoculating the Selenite-F broth tubes, *Salmonella* were found. The preenrichment incubation period of 24 hours was established as an essential step in the

procedure for isolating *Salmonella* from yeast by the following tests which were repeated three times.

A 1:10 suspension of the yeast was incubated at 30 C and aliquots were removed at 2, 4, 6, 8, and 24 hours and inoculated into Selenite-F and tetrathionate broths. The broth cultures were streaked onto brilliant green, S-S, and bismuth sulfite plates after 18 to 20 hours' incubation. No *Salmonella* were found at the 2-, 4-, and 6-hour intervals but growth of *Salmonella* appeared after 8 hours' incubation in only 1 of the samples examined. All were positive after 24 hours at 30 C.

Experiments were worked out to establish the upper and lower range of *Salmonella* contamination in the yeast. Aliquots of a 1:10 yeast suspension were pipetted into 5 replicate test tubes in amounts to give equivalent weights of 2, 1, 0.5, and 0.25 grams of yeast. Sterile 1 per cent yeast water was added to bring the volume in each tube to 20 ml and the suspensions were incubated for 24 hours at 30 C. A loopful from each suspension was streaked directly onto brilliant green, S-S, and bismuth sulfite agar plates; and 1 ml from each tube was inoculated into Selenite-F and tetrathionate broths. The broth cultures were streaked after 18 to 20 hours' incubation onto selective agar plates. *Salmonella*, Group C_1, was found in the 2 g and 1 g samples but not in the 0.5 g samples, which indicated a range of more than one but less than 2 *Salmonella* per gram of yeast. The plates streaked directly from the yeast suspension showed a heavy growth of lactose fermenters and paracolons. *Salmonella* were found on only 5 of the 60 plates streaked (8 per cent). In comparison, 45 per cent of the plates streaked from the Selenite-F and tetrathionate broth cultures were positive for *Salmonella*. No explanation is offered for the anomalous failures of *Salmonella* in yeast to grow in Selenite-F or tetrathionate broth without preenrichment incubation. It is not likely that the low *Salmonella* content in dried yeast played a major role in these failures, inasmuch as the sensitivity of the method for egg products readily permits the detection of *Salmonella* in MPN values as low as 0.1 per gram.

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Summary

An approximation of the extent of *Salmonella* contamination in eggs and egg products may be determined by the use of the multiple dilution method described. This procedure requires 5 replicate tubes of Selenite-F cystine enrichment broth for each decimal dilution, subsequent plating to selective solid media and the application of most probable number tables to the positive tubes in the series.

No single plating medium will allow satisfactory isolation of all species of *Salmonella*, but by using an enrichment medium and subculturing to more than one selective or differential agar, increased sensitivity in detecting the presence of *Salmonella* organisms in food products is obtained.

The quantitative method has been applied to the estimation of the most probable numbers of *Salmonella* in dehydrated whole egg, frozen and liquid whole egg, frozen, liquid and dried egg albumen, and frozen and liquid egg yolk. Qualitative methods for the presence of *Salmonella* in precooked frozen meats, poultry and vegetables, and inactive dried yeast are described.

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